

EVOLUTION AND DISTRIBUTION OF RUBELLA VIRUS GENOTYPES

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Rubella virus is a sole member of Rubivirus group of Togaviridae and its genotypes has been classified in 2004 at WHO meeting. They are genotype 1B, 1C, 1D, 1E, 1F, 2A and 2B as confirmed and 1a, 1g and 2c as provisional. They have unique chronological and geographical characteristics. Genotype 1a worldwide distributed in 1960s and 70s, however almost disappeared since 1980. Genotype 1B mainly has distributed in Europe and genotype 1C in North and South American continents. Genotype 1D has distributed mainly in Asia and genotype 1F is restricted in China. Genotype 1E looks to be derived from genotype 1D and recently becomes to be predominant circulating one worldwide since 1997. Genotype 1g looks to be derived from genotype 1B and distributes in Europe and Americas. Genotype 2A was restricted in China and 2B in Eurasia and Africa. Genotype 2c was found in Russia. Molecular epidemiological study of rubella virus genotype could reveal the transportation of rubella virus from a country to another country. It will greatly help to make a effective plan of rubella immunization program to eliminate and eradicate for a certain country.

Ancestor dating analysis resulted in 1942-46 for virus strains in genotype 1 and 1840 for those in genotype 2.

By these analysis shift of major prevailing genotype of rubella virus may happened in the history of this diseases, at least from genotype 2 to genotype 1 and genotype 1a to genotype 1E.

As rubella has no relating animal viruses as far as studied, this evolution and emergence is very curious to be known in the future.

Molecular epidemiological analyses of recent Japanese encephalitis virus isolates from swine in Japan

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Abstract

Japanese encephalitis (JE) caused by Japanese encephalitis virus (JEV), is the most important viral encephalitis in Asia. Approximately 50,000 cases with 10,000 deaths, mostly among children, are reported annually, mainly in China, Southeast Asian countries and India. In Japan, the JE case number has dramatically deduced and less than 10 cases are reported annually since 1992. However, a high percentage of native pigs are seroconverted to JEV every year in most of the regions in Japan. Thus, JEV still circulates in Japan, and we are even now exposed to the JEV threat. In order to define the characteristics of JEV in East Asia, we started a JEV surveillance in pigs and humans, in collaboration with 12 prefectural institutes since 2002.

Seven, one and three JEV isolates from swine serum samples, in different six prefectures, were obtained in 2002, 2003 and 2004. Nucleotide sequences of the E gene of the 36 JEV strains were analyzed, and all the 13 new isolates were classified into genotype-1. The 28 JEV strains belonging to genotype-1 appeared to be grouped into 2 clusters, and the 13 new isolates were included in the newer cluster with other 11 strains isolated after 1992. Isolation of 2 JEV strains with different nucleotide sequences, in the same pig farm on

the same day, was also observed in the phylogenetic tree.

The nucleotide sequence of 3'-NTRs of the 10 JEV strains belonging to genotype-1 were determined and compared, with those of 5 strains belonging to genotype 1-4. Several peculiar deletions to each four genotypes were found in the alignment. The 9 new isolates and Ishikawa strain showed common five deletions with a preliminary code (no.of nucleotides deleted) of a(2)-b(13)-c(1)-d(1)-e(2), and one of 9 new isolates showed a new deletion in the variable region, which extended from one to ten nucleotides in code-c.

Furthermore, neutralizing antibody response to Beijing-1 strain, and the neurovirulence and neuroinvasiveness in DDY mice, among a current vaccine strain (Beijing-1) and recent JEV isolates from swine were also examined.

As a result, further analyses should be needed to elucidate the subject.

A deletion of 13-15 nucleotides in the 3'-untranslated region of Japanese encephalitis virus genome found in recent isolates induces growth-restriction in cultured cells

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<Objective> Japanese encephalitis cases have decreased in these few decades, but serological surveys have indicated that Japanese encephalitis virus (JEV) still frequently circulates in Japan. One possible mechanism to explain these situations is an attenuation of JEV strains in nature. It has been reported that some recent isolates of JEV contain about 10 nt deletions in the 3'-untranslated region (UTR) in their genomes. Here, we studied effects of a deletion in the 3'UTR on growth property in cultured cells and the pathogenicity in suckling mice.

<Materials and Methods> Based on alignment data using sequences of the recent isolates and the prototype Nakayama strain, 13 or 15 nt deletions were introduced into the infectious cDNA clone of Nakayama strain (Mut13 or Mut15, respectively). *In vitro* transcribed RNA was electroporated into BHK, C6/36, PK15 and IMR32 cells. These cells were compared with those obtained with the wild type, in yields and the number of cells producing progeny virus. For *in vivo* study, 20 µg of RNA was inoculated into 4- to 6-day-old suckling mice via the intracerebral route.

<Results> Comparison of the yield from RNA-electroporated C6/36 cells indicated that Mut13 RNA did not provide detectable yields and Mut15 RNA provided less yields than the wild type until 72 h post electroporation (hpe). In other cell species, Mut13 RNA and Mut15 RNA provided less yields than the wild type only until 24 hpe: the yield obtained with Mut13 RNA was less than that obtained with Mut15 RNA. The number of cells producing progeny virus was much lower in Mut13 than that of Mut15 and the wild type. The virus recovered from Mut13 RNA-electroporated BHK cells at 24 hpe maintained the less growth property in C6/36 cells, but those obtained at 48 and 72 hpe did not. All mice inoculated with Mut15 RNA or the wild type RNA died until 8 days post inoculation (dpi), whereas 91% of mice inoculated with Mut13 RNA survived for 21 days. Interestingly, the replication of Mut13 RNA in the brain was demonstrated at 7 dpi by RT-PCR.

<Discussion> The introduction of 13-15 nt deletions in the 3'UTR of JEV genome induced growth restriction in mammalian cells and much higher in mosquito cells. In addition, Mut13 RNA was less pathogenic in suckling mice than the wild type. However, the recovery of the growth capacity during cultivation of Mut13 RNA-electroporated cells suggests that the mutant virus may acquire some further mutations to compensate the effect of deletions in the 3'UTR.

Japanese encephalitis virus (JEV) isolation from mosquitoes and inhibitory effect of RNAi on JEV infection

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野外蚊からの日本脳炎ウイルス(JEV) 分離及び RNAi による JEV 感染増殖阻害

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Abstract:

JE viruses (JEV) still spreads in Japan, even though JE cases in Japan are less than 10 in each year. However, Japanese government decided in 2005 that the vaccination to JEV is unnecessary. Is that reasonable? It is important for the JE virologist to clarify the real situation of virus distribution in Japan. Last several years, we have tried to isolate virus from the mosquitoes in Ishikawa area of Japan. In the summer of 2005, we collected 1759 mosquitoes and homogenized them, 40 mosquitoes in each sample. RNAs were extracted from those samples. And then we performed RT-PCR and got seven PCR-positive samples. To isolate viruses from the PCR-positive samples, we used Vero cells. Finally we could isolate a virus. By the sequencing analysis, we show that new isolate is genotype I.

In addition, it is also important to develop new antiviral reagent against JEV. For that, we used RNAi method. Here we used several siRNAs for the experiment. Viral reproduction in Vero cells was markedly decreased to 7-10 % of control in the presence of 250 nM siRNAs including JCR and JRi. In mouse experiment, the injection of 5 μ g/g pJRi which expresses the siRNA (JRi) in the cells protected mice 80% from the death by JEV infection and replication. The results indicated that siRNAs have the inhibitory effect on JEV replication *in vivo* and *in vitro*. Therefore RNAi procedure could be used as an antiviral method against JEV or other flaviviruses.

Nucleolar protein B23 interacts with Japanese encephalitis virus core protein and participates in viral replication

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Japanese encephalitis virus (JEV) core protein is detected not only in the cytoplasm but also in the nucleoli of infected cells. We previously showed that a mutant JEV, of which the core protein does not localize in the nucleoli, impaired viral replication in mammalian cells and neuroinvasiveness to mice.

In this study, we identified a nucleolar phosphoprotein B23 as a host protein interacted with the core protein of JEV but not with that of dengue virus. However the core protein of the mutant JEV did not associate with B23. The region binding with JEV core protein was mapped to amino acid residues 38 to 77 of B23. Upon JEV infection, some fraction of B23 was translocated from the nucleoli to the cytoplasm, and cytoplasmic B23 was colocalized with the core protein of the wild-type JEV but not with that of the mutant JEV. And cytoplasmic B23 formed some foci with core protein and other viral proteins only in the cells infected with wild-type JEV. Furthermore, overexpression of deletion mutants of B23 impaired JEV replication as the dominant negatives. These results suggest that B23 plays important roles in the intracellular localization of the core protein and replication of JEV.

Effects of tunicamycin treatment on the growth of Japanese encephalitis virus in BHK cells.

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Oligosaccharides are an integral component of many viral envelope and eukaryotic cell surface proteins which may play significant roles in specific recognition phenomena and influence protein folding and conformation. It has been reported that the envelope (E) protein of Japanese encephalitis virus (JEV) has a single N-linked glycosylation site, and reduced ability to produce extracellular E protein in the presence of tunicamycin (TM). The role of carbohydrate side chain of the JEV E protein, however, is not well understood on its antigenic structure and biological functions. We studied the effect of TM treatment on the growth of JEV in BHK cells. At the concentration of 2.5 µg/ml of TM in the medium, hemagglutinating (HA) activity and infective titer of JEV in BHK cell culture supernatant were markedly reduced at 48 h post infection: 7,311 HA units and 7.1×10^9 p.f.u. per mg of protein, and 105 HA units and 6.9×10^4 p.f.u. per mg of protein were obtained for the control and TM-treated viruses, respectively. Virus specimens were separated by 10% SDS-PAGE, and then transferred to PVDF membrane. The blot was blocked and reacted with hyperimmune mouse sera against JEV E protein. The protein bands were detected by PAGE at a molecular mass (M_r) of approximately 180 K, 110 K and 54 K, and 165 K, 108 K, for the control and TM-treated virions, respectively. On the other hand, the bands corresponding to the E protein were detected by immunoblotting at M_r of approximately 185 K, 55 K, and 30 K, and 170 K, 38 K, and 35 K, for the control and TM-treated virions, respectively. A protein migrating at M_r of 185 K to 170 K corresponding to E protein trimer was detected in both control and TM-treatment, whereas a protein migrating at M_r of 55 K corresponding to E protein monomer was detected only in the control. By analyzing the reactivity of each of the 9 monoclonal antibodies with the E protein, TM treatment influenced maintaining of the E protein conformation and reduced their reactivity. In conclusion, TM treatment inhibits the addition of carbohydrate side chain in the JEV E protein, and influences proper maturation of E protein leading to produce fully infectious virus in BHK cells.

Involvement of conserved region of flavivirus prM protein in virus particle budding

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Enveloped viruses acquire their lipid envelopes by budding through the plasma membrane or the membrane of an intracellular organelle. Flaviviruses are assembled to bud into the lumen of the endoplasmic reticulum (ER) and are secreted through the vesicle transport pathway. Virus envelope protein prM and E are considered to play important roles in budding and secretion process. However, little is known about the details of the mechanism of the process. In this study, we investigated the involvement of conserved region in flavivirus prM protein in virus particle budding, using subviral particles (SPs) system and infectious cDNA clone.

In SPs system of tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV), mutations were introduced into the conserved regions of prM proteins, and the effects of these mutations on SPs secretion were examined. Secretion of SPs was drastically reduced by the prM mutations and viral envelope proteins accumulated intracellularly. Co-immunoprecipitation assay showed that the interaction between prM and E protein was not affected by the mutations. As observed by immunofluorescence microscopy, viral envelope proteins with the mutations in prM were scarce in the Golgi complex, and accumulated in the ER. Electron microscopic analysis of cells expressing the mutated prM revealed that many tubular structures were present in the lumen, indicating that budding process of virus particle was affected by the mutations in the conserved region in prM. Furthermore, to investigate the effect of the mutation on live virus, the mutation was inserted into TBE virus infectious cDNA clone and secretion of infectious virus was investigated. Infectious virus particles with prM mutations produced fewer progeny virus than native virus as observed in SPs system. These data suggest that conserved region in prM plays a crucial role in the virus budding process.

Effect of complement on dengue antibody-dependent infection enhancement assay

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Antibody-dependent enhancement (ADE) of infection is one mechanism relating to dengue severity. We have generated and characterized mouse anti-dengue type 2 or type 4 virus (DENV2 or DENV4) monoclonal antibodies (MAbs). So far, our results indicated that all MAbs showing ADE activities also showed neutralizing (NEUT) activities. In addition, we found that inclusion of rabbit complement in our ADE assay system (virus-antibody-cell mixture) dramatically reduced ADE activities which were shown in the system without using complement. In the present study, we further analyzed effects of complement in more detail.

MAbs used were those generated from DENV2- or DENV4-immune mice and showing type-specific and/or crossreactive ADE activities. For the ADE assay, U937 cells were infected with a mixture of virus (DENV2 or DENV4) and MAb in the absence/presence of rabbit complement or fresh human serum. After incubation at 37°C for 6 days, cells were immunochemically stained to count infected cells.

The ADE activity decreased with increasing concentrations of the complement. Heat inactivated complement or the complement pretreated by anti-complement (C1q, C3, factor B) antibodies did not reduce ADE activities. Similar dose responses and effects of heat-inactivation and anti-complement antibodies were shown when fresh human sera were used, suggesting that the complement contained in human sera is a critical factor to control ADE activity. The effect on ADE activity varied according to the serum sample. Some sera showed less reducing effects on crossreactive than specific ADE activities.

These results suggest a potential mechanism of disease severity based on the level of complement in the circulation that can control the two conflicting functions of antibody, neutralizing and ADE activities. The less reducing effects on crossreactive than specific ADE activities may explain epidemiological evidence showing severer cases in the secondary heterotypic than homotypic infections.

Labeled avidin-biotin ELISA using anti-bat IgG rabbit serum for detection of Yokose virus antibody in bat sera

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Enzyme-linked immunosorbent assay (ELISA) using biotin-labeled rabbit anti-bat IgG was applied to detect antibody in bat sera. At first, Yokose virus (YOKV), which was isolated from microbats in Japan and belongs to the genus *Flavivirus*, was chosen as a virus antigen. Purified Oita-36 strain of YOKV, biotin labeled anti-bat IgG conjugate and horseradish peroxidase (HRP)-avidin were employed in the assay. To obtain positive sera of bats against YOKV, immunization of bats was conducted.

By use of this labeled avidin-biotin (LAB)-ELISA, serological surveillance was conducted with the sera collected in Thailand and the Philippines, and the sera supplied from Malaysia. Of the total 186 collected serum samples, 151 samples were sufficient in volume and assayed by ELISA. One sample from the Philippines (2.7%), five from Malaysia (19%), none from Thailand had detectable antibodies. Serum samples were also tested by neutralization test (NT), and the correlation rate between ELISA and NT was 0.79. These data suggest that Yokose virus is distributed not only in Japan but also in other Asian countries. To examine the specificity of ELISA, ELISA substituting the antigen to Japanese encephalitis virus, which was widely distributed in South-East Asian countries, was conducted. ELISA with JEV antigen reacted with the positive serum against YOKV, but the titer was much lower than homologous ELISA titer with YOKV antigen. This suggests the specificity of the ELISA for detecting anti-YOKV antibody in bat sera. And, the ELISA is available at least for the screening of antibodies against flaviviruses.

This ELISA system would be applied to detect the antibodies against viruses belong to the other virus families by substituting only viral antigen.

Seroepidemiological study on chikungunya virus infection in Southeast Asia and Pacific region.

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[Introduction]

Chikungunya virus (CHIK) is a member of the *Alphavirus* genus of the family *Togaviridae*. It is widely distributed in the Southeast Asia, Africa and the western Pacific islands. Due to the similarity of clinical symptoms with dengue fever and long interval between outbreaks, the laboratory diagnosis of CHIK is not routinely done. This study aimed to analyze the positive ratio of CHIK among the cases which were clinically suspected/diagnosed as dengue virus infection in five countries in Southeast Asia and the Pacific region.

[Patients and Methods]

Three hundred and five serum samples collected from dengue suspected patients from the year 2001 to 2005 were provided from Fiji, the Philippines, Indonesia, Bangladesh and Sri Lanka. As serological diagnoses, CHIK IgM-capture ELISA and CHIK IgG-indirect ELISA were carried out.

[Results]

Fifteen cases (4.9%) were CHIK IgM positive, and 41 cases (13.2%) were CHIK IgG positive as a total. The highest CHIK IgM positive ratio, 11.1%, was obtained from the Philippines, and the lowest ratio, 0%, was from the Bangladesh. The highest CHIK IgG positive ratio, 25.0%, was obtained from Indonesia and followed by the Philippines (14.3%), and the lowest ratio, 4.9%, was from Fiji.

[Discussion]

Although CHIK is believed not exist in Fiji, another member of the Semliki Forest antigenic complex, which is Ross River Virus (RRV), has been reported as an outbreak in 1970th. Therefore, These CHIK sero-positive cases from Fiji might be due to the recent RRV infection.

VECTOR COMPETENCE OF JAPANESE SALT MARSH MOSQUITO, *CULEX MODESTUS INATOMII* AGAINST TWO NEW YORK STRAINS OF WEST NILE VIRUS

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West Nile virus (WNV) infection and transmission ability of an autogenous mosquito, *Culex modestus inatomii* that was found in salt marsh in Japan and Korea were observed. The larvae were collected in saltmarsh water near the gulf of Osaka. The mosquito colony was established with 0.5% salt water for rearing the larvae at laboratory. Two strains originated from birds and mosquitoes in New York stocks of WNV were used. Both of the viral genomes were individually detected from orally infected mosquitoes 10 days post infection. And also their positive thorax suggested virus transmission. Oviposited female mosquitoes were used for the transmission experiment due to the autogenous characteristic. The mosquitoes 10 days post intrathoracically infection had a chance to engorge blood meals from 8-week mice, individually. Some mice showed symptom of the infection after 4 to 8-day infection. Brains and spleens of the mice were examined for the viral genome using RT-PCR. Out of 8 mice, seven mice showed positive for the viral genome. *Culex inatomii* is presumed to be a possible vector of WNV same as *Culex modestus modestus*, the vector in Europe. Distribution of the habitat may be limited, because the larvae grow well in salt marsh water than pure water. The habitat area however may be enlarged temporarily, if salt marsh expands with the rise of sea level due to the global warming.